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Expression of Integrin $\alpha 6\beta 4$ in Junctional Epidermolysis Bullosa

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The integrin $\alpha 6\beta 4$ is a member of the integrin family of adhesion receptors. The integrin $\alpha 6\beta 4$ is preferentially expressed in stratified squamous epithelia, where it is localized in hemidesmosomes. A reduced number of rudimentary hemidesmosomes is often found in skin from patients with junctional epidermolysis bullosa (JEB). In this study we have investigated the expression of $\alpha 6\beta 4$ in skin specimens of patients with junctional (one non-lethal, two lethal) and dystrophic (two) epidermolysis bullosa, using immunofluorescent (IF) staining with five different monoclonal antibodies against the $\alpha 6$ and $\beta 4$ subunits. The intensity of IF staining of the integrin $\alpha 6\beta 4$ and bullous pemphigoid antigen (BPA) was unreduced along the epidermal basement membrane zone (EBMZ) of all EB patients, compared to that in skin of healthy human controls. However, in the skin of two patients with lethal (Herlitz) JEB, who did not express GB3, IF staining of integrin $\alpha 6\beta 4$ and BPA showed a "stitchy" discontinuous linear pattern along the EBMZ with interruptions at the borders of adjoining basal keratinocytes. The same results were obtained by immunoelectron microscopy. They corresponded with freeze-induced partial cell detachment from the basement membrane at the ultimate baso-lateral edge of the basal keratinocytes in lethal JEB skin. The basal lamellipodia at that location almost completely lacked tonofilaments and hemidesmosomes. Furthermore, in JEB there was a split between the intra- and extracellular epitopes of the integrin $\alpha 6\beta 4$ receptor, whereas the integrin remains intact in salt-split skin. This suggests that the defect is in $\alpha 6\beta 4$ itself or perhaps its ligand. *J Invest Dermatol 99:489–496, 1992*

Junctional epidermolysis bullosa (JEB) represents a subgroup of hereditary mechanobullous diseases in which there is always a cleavage plane in the lamina lucida of the epidermal basement membrane zone (EBMZ). Most patients with JEB have ultrastructurally defective hemidesmosomes, e.g., absent sub-basal densa plates and rudimentary attachment plaques [1]. The antigenicity of the EBMZ is altered in patients with JEB: the lamina lucida antigen BM-600/nicein identified by the antibodies AA3 [2] and GB3 [3] is reduced or absent. JEB patients also do not express the antigen identified by monoclonal antibody(ies) (MoAb) 19-DEJ-1, which is associated with the hemidesmosomal anchoring filament complex [4]. The bullous pemphigoid antigen (BPA), which is exclusively localized in the plaque of the hemidesmosome [5,6], is normally expressed in JEB [7,8] and no gross abnormalities of the 230-kD BPA gene can be detected by Southern blot analysis in these patients [9]. It is still unknown which antigenic determinant of the hemidesmosomes is defective in patients with JEB. A recently recognized member of the integrin superfamily of adhesion receptors, the integrin $\alpha 6\beta 4$ [10,11] might be a candidate.

Integrins are a superfamily of cell-surface receptors [12], consisting of noncovalently linked heterodimers of α and β subunits, which mediate cell-cell and cell-matrix adhesion. At least 13 α [13] chains and seven β chains [14] have been recognized, of which only $\alpha 6\beta 4$ is exclusively associated with keratinocyte-basement membrane adhesion [15]. The evidence that integrin $\alpha 6\beta 4$ is preferentially located in hemidesmosomes was recently independently obtained by several laboratories and was based on immunoelectron microscopy (IEM) on cultured epithelial cells [16,17] and epithelial tissues [15,18–20] using antibodies against the $\alpha 6$ and $\beta 4$ subunits. In support of this, it was shown that $\alpha 6\beta 4$ co-distributes with the hemidesmosome component 230-kD BPA [15,17]. Integrin $\alpha 6\beta 4$ polarizes within 12 h to the basal surface of cultured keratinocytes [21], which appears to be calcium dependent [22]. Anti-$\beta 4$ antibodies strongly inhibit keratinocyte adhesion to various substrates [21,22]. Recently, Jones et al demonstrated that anti-$\alpha 6\beta 4$ antibodies prevent hemidesmosome assembly and alter the IF pattern of 230-kDa BPA (and probably integrin $\alpha 6\beta 4$) [19]. These studies suggests that the integrin $\alpha 6\beta 4$ may act as a nucleating center for hemidesmosomes.

In a previous study by Nazzaro and colleagues [23,24], $\alpha 6$ subunit (GoH3 [25]) was found to be normally present along the EBMZ in patients with JEB (two lethal, one non-lethal). Fine et al recently

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Abbreviations:
BPA: bullous pemphigoid antigen
BMZ: basement membrane zone
Casbl: cascade blue
EB: epidermolysis bullosa
EBMZ: epidermal basement membrane zone
EM: electron microscopy
FITC: fluorescein isothiocyanate
HRP: horse radish peroxidase
IF: immunofluorescence
IEM: immunoelectron microscopy
IgG: immunoglobulin G
JEB: junctional epidermolysis bullosa
MoAb: monoclonal antibody(ies)
PBS: phosphate-buffered saline
PLP: periodate-lysine-paraformaldehyde

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Table I. Summary of the Examined MoAb Against Human Integrin $\alpha6\beta4$

MoAb	Titer	Host	Ig Isotype	Subunit	Epitope Localization
450-11A	50	Mouse	IgG1	$\beta4$	Intracellular
450-10D	50	Mouse	IgG2a	$\beta4$	Intracellular
439-9B	50	Rat	IgG2	$\beta4$	Extracellular
450-30D	50	Mouse	IgG1	$\alpha6$	Extracellular
GoH3	50	Rat	IgG2a	$\alpha6$	Extracellular

reported in an abstract that the expression of the integrin $\alpha6\beta4$ is unreduced in each of over 60 JEB patients [26]. However, no details are available concerning the spatial distribution of $\alpha6\beta4$ in relation to BPA, GB3, and laminin in JEB skin.

In this study we report on the expression of both the $\alpha6$ and $\beta4$ subunits, using five MoAb against different intra- and extracellular epitopes of the integrin $\alpha6\beta4$ complex in patients with junctional and dystrophic epidermolysis bullosa (EB).

MATERIALS AND METHODS

Patients Five patients with EB were studied: three with JEB and two with dystrophic EB. The three patients with JEB included a 2-month old female (patient 1) and a 6-month old male patient (patient 2) suffering from the lethal (Herlitz) variant, and a 39-year old male patient (patient 3) with the non-lethal variant (synonym: generalized atrophic benign EB). The two dystrophic EB patients included a 6-month old female patient (patient 4) with the dominant (Cockayne-Touraine) variant, and a 7-year old female patient (patient 5) with the recessive generalized mitis (non-mutilating) variant. The case histories of patients 1 and 5 have been published [27,28]. Diagnosis was established in each patient on the basis of clinical findings, family history, immunofluorescence mapping, and electron microscopy (EM). All three patients with JEB had a reduced number of hypoplastic hemidesmosomes lacking the subbasal densa plate in clinically uninvolved skin. Skin specimens of three healthy adults and a 1-year old child served as controls.

Skin Specimens For the IF and EM studies we used 4-mm punch-biopsy specimens, obtained under local anesthesia with 2% lidocaine from apparently uninvolved skin from the extremities of the EB patients. In addition, a biopsy of lesional skin was obtained. Skin specimens of three healthy adults and a 1-year old child served as controls. The skin specimens were sn/LRSC, nap frozen in liquid nitrogen and stored at −70°C.

For the in situ localization of integrin $\alpha6\beta4$ in a model split of the EBMZ, we used skin tissue obtained from breast reductive surgery. Splitting of the EBMZ was achieved by a slightly modified incubation technique with NaCl as originally described by Scaletta et al [29], introduced as a tool for antigen localization by Gammon et al [30]. The skin specimens were incubated for 24 h in 1.0 M NaCl at room temperature, followed by washing for 30 min in PBS (vide infra), and snap frozen in liquid nitrogen. Immunofluorescence antigen mapping showed that laminin was always in the blister floor and the BPA in the blister roof.

Antibodies Characteristics of the monoclonal antibodies (MoAb) against the integrin $\alpha6$ and $\beta4$ subunits are summarized in Table I. MoAb 450-11A, 450-10D, 439-9B, and 450-40D were kindly provided by Dr. S.J. Kennel [31,32]. MoAb GoH3 has been characterized in a previous study [25]. The GB3 marker for JEB was obtained through the courtesy of Dr. J.-P. Ortonne [33]. Other primary antibodies included polyclonal rabbit anti-laminin (Telios, CA) and biotinylated human IgG autoantibody against the BPA [34].

Secondary antibodies included biotinylated horse anti-mouse IgG (Vector Labs, CA), biotinylated goat anti-rabbit IgG (Protos Labs, CA), fluorescein isothiocyanate (FITC)-conjugated goat anti-rat immunoglobulin G (IgG) (SBA, Alabama, USA), and cascade blue (Casbl)-conjugated goat anti-rabbit IgG (Molecular Probes, OR). The binding of biotinylated antibodies was visualized by incubation with either FITC-conjugated extra-avidin (Biomakor) or LRSC (lissamine rhodamine)-conjugated streptavidin (Jackson ImmRes, PA).

Immunofluorescence (IF) Procedures Cryosections of 4 μm thickness were mounted on silane-coated slides, air dried for 30 min by using a fan, and stained for 30 min with the properly diluted primary antibodies. After three washes for 5 min each in phosphate-buffered saline (PBS; 0.01 M; pH 7.3), the sections were incubated for 30 min with the appropriate FITC- or biotin-labeled secondary antibody. In case of biotinylated secondary antibody, the sections were washed again in PBS and incubated for 30 min with either extra-avidin/FITC or streptavidin/LRSC conjugate. A typical triple antigen-labeling experiment (Fig 1*B*) consisted of the following seven incubation steps: 1) mouse IgG anti-GB3, 2) horse IgG anti-mouse IgG/Bio, 3) streptavidin/LRSC, 4) rat IgG anti-439-9B, 5) goat Ig anti-rat IgG/FITC, 6) rabbit IgG anti-laminin, 7) goat Ig anti-rabbit IgG/Casbl.

A number of control staining experiments were performed to ensure the specificity of double and triple antigen-labeling procedures. These control experiments included purposely cross-reactive immunostaining steps that confirmed the expected "false" fluorescent color profiles of the basement membrane epitopes.

Sections were mounted in PBS-glycerol (50%; pH 7.5). For photomicrography, the sections were mounted in PBS-glycerol containing p-phenylenediamine (1.5 mg/ml).

The sections were examined with a Leitz Orthoplan microscope equipped with both high-pressure Xenon (XBO-75W) and Mercury arc (HBO-50W), and Ploemopak unit for incident-light excitation. For selective excitation and emission, varying combinations of filter blocks (Leitz/Leica) were used including the L3 block (green), the A2 block (blue), the TXR block (red), and a newly developed FITC/TXR dual-band block (Omega Optical Inc., Vermont) for simultaneous green and red fluorescence.

Photomicrographs were made with the Vario Orthomat-2, using Fujichrome 400 ASA color reversal film. Photomicrography of multiple-stained tissue sections was performed by a specially developed selective double- or triple-wavelength recording technique involving geometrical (optical) shift adjustment.

Electron Microscopy Biopsies from uninvolved skin were fixed with 2% glutaraldehyde in 0.1 M phosphate buffer, stained with 2% osmium tetroxide, embedded in epon, sectioned, and counterstained with uranyl acetate. The ultra-thin sections were examined with the Agashi 002A electron microscope.

Immunoelectron Microscopy Cryosections of 15 μm thickness were mildly fixed for 3 min in 0.015% periodate-lysine-paraformaldehyde (PLP) reagent, washed in PBS and stained en bloc for 30 min with 450-10D ($\beta4$) primary antibody. After washing in PBS, sections were incubated for 30 min with the HRP-labeled rabbit anti-mouse Ig conjugate, washed again, and treated for 30 min with the HRP-labeled goat anti-mouse Ig conjugate. The sections were washed, postfixed in 0.5% glutaraldehyde in phosphate buffer (0.1 M, pH 7.4), washed again, and developed with 0.05% 3,3′diaminobenzidine tetrahydrochloride (DAB; Sigma) in PBS with 0.03% hydrogen peroxide. The sections were further processed for electron microscopy as described above, omitting the step for counterstaining.

RESULTS

IF of Normal Human Skin The results of analysis of $\alpha6\beta4$ expression in skin tissue sections by immunofluorescence are listed in Table II. In normal human skin, staining with MoAb against integrin $\alpha6$ and $\beta4$ subunits produced a continuous linear fluorescence pattern along the EBMZ (Fig 1*A*). In addition, MoAb against the extracellular epitopes of the $\alpha6$ (450-30D, GoH3) and $\beta4$ subunit (439-9B) focally stained the lateral and apical borders of basal

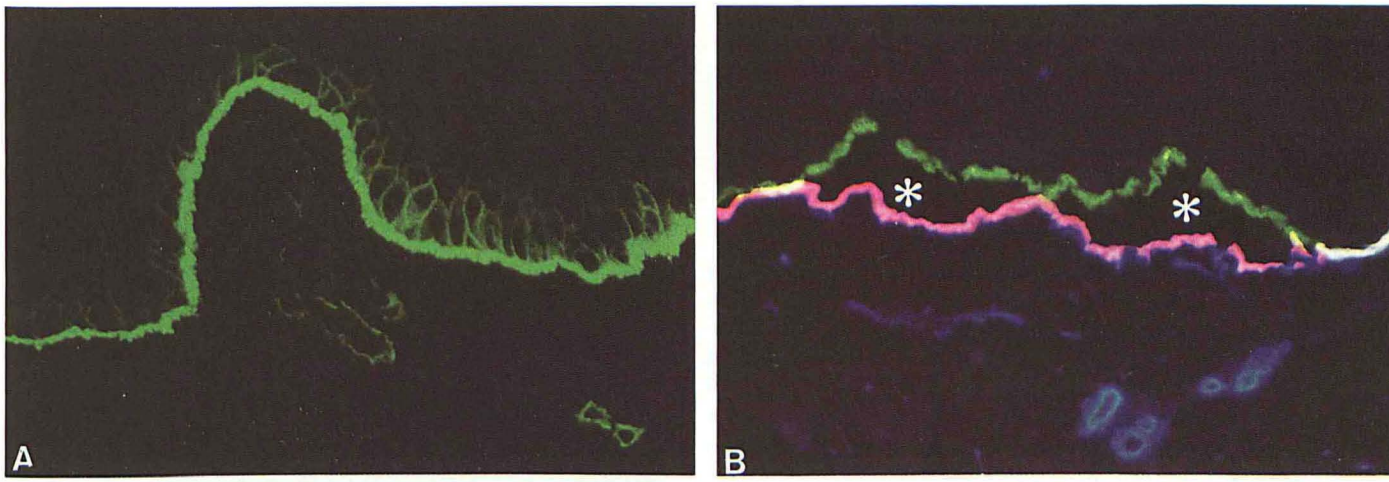

Figure 1. IF staining of cryosections of (*A*) intact control skin, and (*B*) salt-split control skin from a healthy person. (*A*) Single IF staining with MoAb 439-9B in intact control skin, showing the presence of the extracellular integrin β4 epitope in a continuous linear pattern at the epidermal basement membrane zone (EBMZ) and at the borders of basal keratinocytes. Note that the β4 epitope is also faintly present in subepidermal bloodvessel walls. (*B*) Triple IF staining for the same β4 epitope (green), GB3-antigen (red), and laminin (blue) in NaCl-split control skin (blister cavity marked by *asterisks*), showing the green fluorescent β4 epitope in the blister roof. The patchy β4 staining pattern is induced by 1.0 M NaCl incubation (compare with *A*). Both laminin and GB3 are localized in the blister floor, yielding the purple-pink staining pattern as a result of superposition of blue fluorescent laminin and red fluorescent GB3. Note that overlap of all three fluorescent markers along the intact EBMZ at the lateral borders of the specimen results in a white color. The ultimate specificity of the triple immunostaining can be judged from the blue fluorescent laminin in subepidermal blood vessel walls that lack the GB3 antigen. The luminal site of these vessel walls additionally show faint expression of the β4 epitope, resulting in a mixture of blue and green fluorescence. Magnification ×500.

keratinocytes in a finely speckled or continuous pattern (Fig 1*A*). This intercellular expression of integrin epitopes varied between specimens and with the type of MoAb used. Furthermore, MoAb against the intracellular part of the β4 subunit (450-11A, 450-10D) produced granular cytoplasmic staining of weak intensity in focal clusters of basal keratinocytes.

Linear staining of α6β4 was also found with varying intensity along the BMZ of epidermal appendices (hair follicles, sebaceous glands, sweat glands, and ducts) and that of some blood vessels, but not at the basement membranes of the mm. arrectores pili. Peripheral nerves, in particular nerve endings associated with mm. arrectores pili and Vater-Pacinian corpuscles, were also stained to various degrees by anti-α6 and -β4 MoAb. The GB3 antibody stained the EBMZ in a broad continuous linear pattern. In addition, GB3 stained the basement membranes of sweat glands and sebaceous glands, but not those of blood vessels.

In salt-split skin, MoAb to the intracellular β4 epitopes exclusively stained the blister roof, whereas MoAb against the extracellular α6 or β4 epitopes additionally produced fine speckles in the blister floor, indicating that the salt-induced split occurred just beneath the integrin. After NaCl incubation, staining for α6β4 was irregular and patchy (Fig 1*B*), which has recently also been reported by Michalaki et al [35]. In triple-staining experiments on salt-split skin, the α6β4 epitopes were localized predominantly in the roof of the blister, whereas laminin and GB3 were found exclusively in the blister floor (Fig 1*B*). Using the dual-band FITC/TXR filter block, we observed that the in non-split areas of the EBMZ the green fluorescent extracellular β4 epitope was clearly localized above

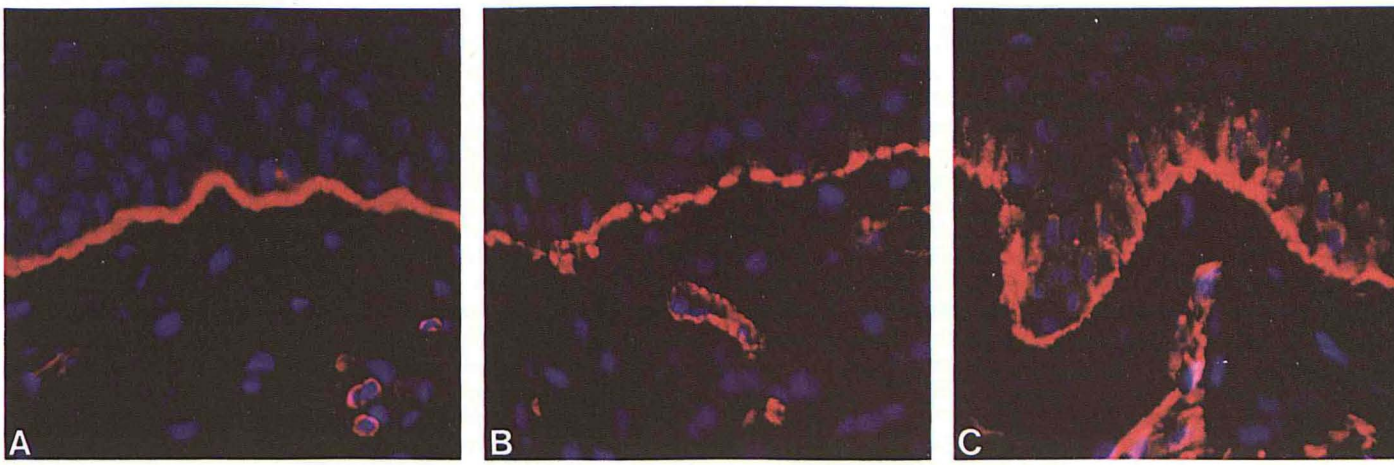

Figure 2. Single IF staining for integrin β4 subunit (MoAb 450-11A) in cryosections of normal skin from (*A*) a healthy human control and from (*B,C*) lethal JEB patients 2 and 1, respectively. The cell nuclei were counterstained with the blue fluorescent DNA marker bisbenzimide. (*A*) Usual linear staining pattern of (α6)β4 at the EBMZ in control skin. (*B,C*) In apparently healthy skin from the lethal JEB patients, the β4 staining pattern is interrupted at the borders of adjoining basal keratinocytes yielding a "stitchy" linear pattern. (*C*) The skin of patient 1 additionally shows focal cytoplasmic expression of the β4 subunit in basal keratinocytes. Magnification ×500.

Table II. IF-Staining Characteristics of the MoAb Against Integrin $\alpha6\beta4$ and Nicein in Skin of Patients with Junctional and Dystrophic Epidermolysis Bullosa and of Healthy Human Controls

MoAb	Patient (number)	CYT[a]	ICS[b]	EBMZ[c]
450-11A ($\beta4$)	Junctional, lethal (1)	+[d]	−[e]	+i[f]
	Junctional, lethal (2)	±[g]	−	+i
	Junctional, non-lethal (3)	−	−	+
	Dystrophic, dominant (4)	±	−	+
	Dystrophic, recessive (5)	±	−	+s[h]
	Normal controls (n = 3)	±	−	+
450-10D ($\beta4$)	Junctional, lethal (1)	+	−	+i
	Junctional, lethal (2)	±	−	+i
	Junctional, non-lethal (3)	−	−	+
	Dystrophic, dominant (4)	−	−	+
	Dystrophic, recessive (5)	−	+	+s
	Normal controls (n = 3)	±	−	+
439-9B ($\beta4$)	Junctional, lethal (1)	+	−	+i
	Junctional, lethal (2)	+	−	+i
	Junctional, non-lethal (3)	−	−	+
	Dystrophic, dominant (4)	−	−	+
	Dystrophic, recessive (5)	−	−	+s
	Normal controls (n = 3)	±	+	+
450-30D ($\alpha6$)	Junctional, lethal (1)	−	±	+i
	Junctional, lethal (2)	−	+	+i
	Junctional, non-lethal (3)	−	+	+
	Dystrophic, dominant (4)	−	+	+
	Dystrophic, recessive (5)	−	+	+s
	Normal controls (n = 3)	−	+	+
GoH3 ($\alpha6$)	Junctional, lethal (1)	−	±	+i
	Junctional, lethal (2)	−	±	+i
	Junctional, non-lethal (3)	−	±	+
	Dystrophic, dominant (4)	−	±	+
	Dystrophic, recessive (5)	−	±	+s
	Normal controls (n = 3)	−	+	+
GB3 (Nicein)	Junctional, lethal (1)	−	−	−
	Junctional, lethal (2)	−	−	−
	Junctional, non-lethal (3)	−	−	+
	Dystrophic, dominant (4)	−	−	+
	Dystrophic, recessive (5)	−	−	+s
	Normal controls (n = 3)	−	−	+

[a] CYT, cytoplasmic staining of keratinocytes.
[b] ICS, intercellular staining of keratinocytes.
[c] EBMZ: epidermal basement membrane zone of intact skin.
[d] +, clearly present (EBMZ, continuous linear) staining.
[e] −, no staining.
[f] +i, interrupted linear staining along EBMZ.
[g] ±, reduced staining.
[h] +s, smooth and thin continuous linear staining along EBMZ.

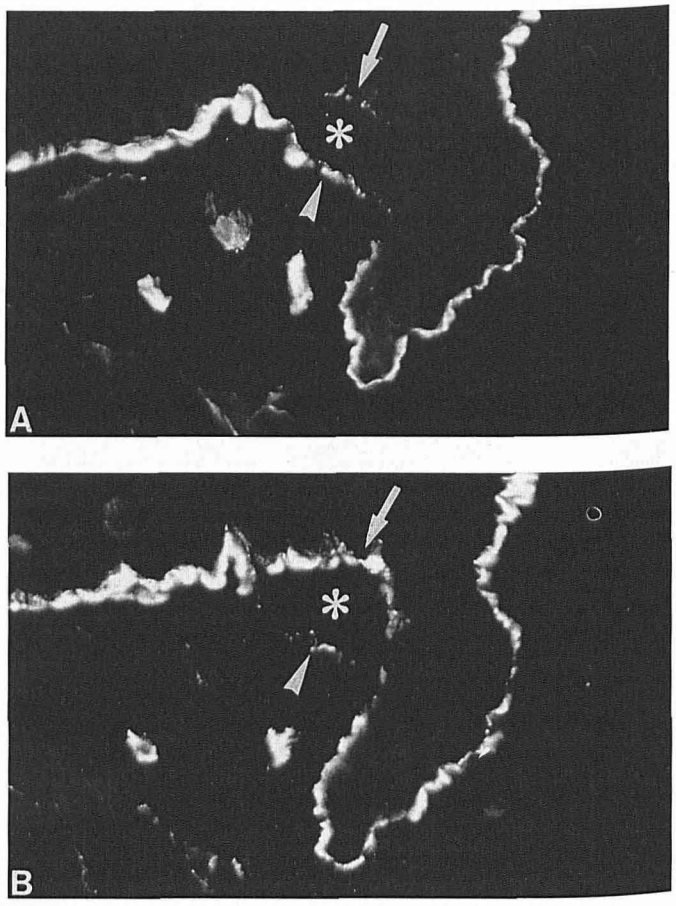

Figure 3. Single IF staining of (*A*) extracellular integrin $\alpha6$ (GoH3) epitope and (*B*) intracellular $\beta4$ (450-11A) epitope in serial cryosections of normal skin from lethal JEB patient 2, showing a tiny blister (*asterisk*) at the follicular BMZ. (*A*) The extracellular $\alpha6$ epitope is mainly expressed in the blister floor (*arrowhead*), whereas in the adjacent section (*B*) the intracellular $\beta4$ epitope is mainly localized in the blister roof (*arrow*). Magnification ×500.

(epidermal site) the blue fluorescent laminin and red fluorescent GB3.

IF of EB Skin There was no staining with GB3 antibody in the skin of the two patients with lethal JEB (patients 1 and 2), but staining appeared to be normal in the skin from the patient with non-lethal JEB (patient 3) (Table II). Staining with MoAb against the $\alpha6$ and $\beta4$ epitopes produced a continuous linear pattern at the EBMZ in all EB patients similar to that in control skin specimens, except for the two patients with lethal JEB (patients 1 and 2) (Fig 2*A*–*C*). In these latter two patients the linear IF pattern of the $\alpha6$ and $\beta4$ subunits was interrupted discontinuous—in patient 2 more clearly than in patient 1—yielding a "stitchy" pattern with small interruptions at regular intervals corresponding with the boundaries of adjoining basal keratinocytes (Fig 2*B*). Examination of serial sections showed that this "stitchy" pattern had a focal distribution and was more distinct at the BMZ of hair follicles. Also the staining pattern of BPA was "stitchy" along the EBMZ in skin of the two patients with lethal JEB (data not shown), whereas the pattern of laminin was normally continuous. In addition, the $\beta4$ subunit was markedly expressed in a fine granular cytoplamic pattern in focal basal keratinocytes of the skin from the patients with lethal JEB (Fig 2*C*). Such an aberrant integrin pattern was not found in the other EB patients. Specifically, $\alpha6\beta4$, BPA, and GB3 were normally expressed in the skin from the patient with non-lethal JEB.

In the clinically uninvolved skin samples from the two lethal JEB patients, tiny blisters were noticed at the BMZ of hair follicles. In these tiny blisters, the extracellular $\alpha6\beta4$ epitopes predominantly lined the blister floor, whereas the intracellular epitopes predominantly remained in the blister roof (Fig 3*A*–*B*). The $\alpha6\beta4$ epitopes lost their antigenicity in older bullae, irrespective of the split level as we observed in biopsies from lesional skin of dystrophic and junctional EB patients. Loss of $\alpha6\beta4$ antigenicity has also been observed in bullae of patients with bullous pemphigoid by Michalaki et al; according to these authors this differentiates bullous pemphigoid from epidermolysis bullosa acquisita [35].

In patient 5, with recessive generalized EB, the IF pattern along the EBMZ appeared as a smooth and thin line, differing from that in control skin and the other EB skin specimens. This smooth IF pattern was observed with both the anti-$\alpha6\beta4$ and GB3 MoAb, as well as with anti-laminin, anti-collagen IV, and anti-collagen VII (LH 7:2) antibodies. Electron microscopy of clinically uninvolved skin of this patient showed reduced keratinocyte projections with loss of anchoring fibrils resulting in a more stretched, less folded basement membrane (data not shown).

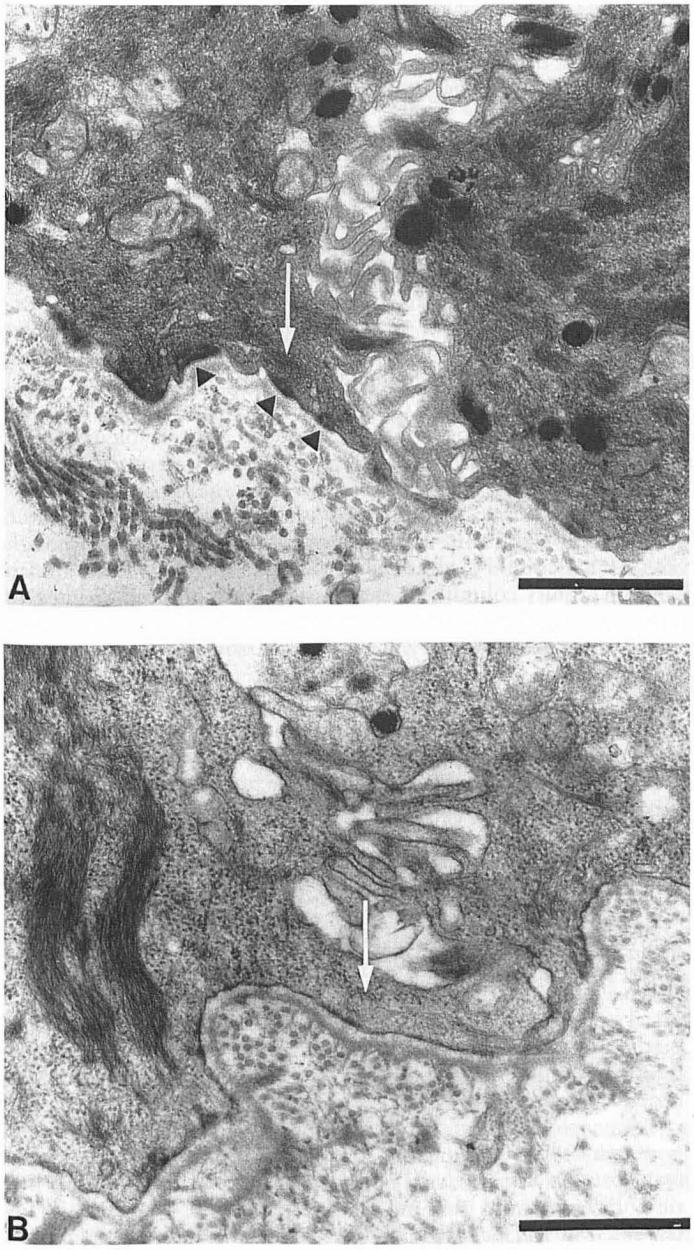

Figure 4. Transmission electron microscopy showing the basement membrane zone of two adjoining basal keratinocytes in (*A*) a healthy human control and in (*B*) a patient with lethal JEB (patient 2). (*A*) Keratinocytes touch each other by interdigitating lamellipodia. The basal keratinocytes anchor to the basement membrane through intermediate tonofilaments (*arrow*) and hemidesmosomes (*arrowheads*), also present in the ultimate basal lamellipodium. (*B*) The basal lamellipodium (*arrow*) in lethal JEB skin almost completely lacks intermediate tonofilaments and hemidesmosomes. *Bar*, 1 μm.

EM of Lethal JEB and Normal Human Skin In uninvolved skin from the two patients with lethal JEB a decreased number of hemidesmosomes was noticed in the electron microscope. The remaining hemidesmosomes had a smaller attachment plaque and lacked the sub-basal dense plate. In the basal keratinocytes a decrease of intermediate filaments (tonofilaments) was noticed, in particular of those projecting towards the inferior pole. These cytoskeletal abnormalities were most pronounced at the periphery of the inferior pole, at the boundary of adjoining basal keratinocytes. Basal keratinocytes normally adjoin at the basement membrane by lamellipodia (Fig 4*A*). In the skin from the lethal JEB patients these lamellipodia almost completely lacked hemidesmosomes and were also devoid of cytoskeletal structures such as intermediate (keratin) filaments (Fig 4*B*). Anchoring filaments, lamina lucida, lamina densa, and anchoring fibrils, however, all appeared structurally normal.

IEM of Lethal JEB and Normal Human Skin β4 (450-10D) was expressed on the basal surface along the EBMZ. It was preferentially confined to hemidesmosome-like structures, which occur more irregularly in JEB skin. In lethal JEB skin the lamellipodia at the cellular boundaries of basal keratinocytes had detached from the lamina densa. At that point the staining for β4 was interrupted (Fig 5*B*). This peripheral cell detachment and β4 interruption was not observed in healthy human control skin (Fig 5*A*).

DISCUSSION

In this study we found that the distribution pattern of the integrin α6β4 and BPA was abnormal along the EBMZ, coinciding with the complete absence of the GB3 antigen in patients with lethal JEB. Specifically, IF staining for the α6 and β4 epitopes and BPA was not continuous along the EBMZ, but was interrupted at the boundary of adjoining basal keratinocytes. IEM revealed that the interruptions in the integrin staining patterns in these patients were caused by focal tissue damage at the ultimate baso-lateral edge of adjoining basal keratinocytes. The tissue damage appears to be caused by freezing and thawing, which was used for IF and IEM preparation, because it was absent in routine EM and non-frozen, fixed tissue samples of uninvolved JEB skin. EM revealed that the basal lamellipodia along the EBMZ at the point where basal keratinocytes meet almost completely lacked cytoskeletal elements and hemidesmosome-like structures. It thus appears that the adherence of keratinocytes in JEB patients who lack expression of GB3 (BM600/nicein) is weakest to the basement membrane at the cell periphery.

In the skin of the patient with the non-lethal type of JEB (patient 3), α6β4 was expressed in a normal continuous linear pattern. The expression of GB3 was also normal in 40% of patients with non-lethal JEB [36]. The overall fluorescence intensity of α6β4 and BPA in both the patients with lethal and the patient with non-lethal JEB in our study was not reduced, despite the decreased number and abnormal structure of hemidesmosomes. It is known from studies of cultured keratinocytes that hemidesmosomal antigens might be expressed in the absence of assembled hemidesmosomes [19,37].

In previous IF studies, Nazzaro et al found no significant alteration in the intensity or distribution pattern of the α3 and α6 subunits stained with MoAb in clinically uninvolved skin of patients with JEB [23]. The expression of the β4 subunit was not investigated. Fine et al reported a normal expression of α6β4 in a series of over 60 JEB patients [26]. Our preliminary study confirms the overall unreduced staining intensity of α6β4 in JEB skin.

The positive expression of both the integrin α6β4 and BPA suggests that neither are primarily defective in JEB. The abnormal hemidesmosomes avoiding the lateral edge of the basal surface of basal keratinocytes suggest another factor that interacts with hemidesmosome assembly. Recently, Krueger et al showed that cultured keratinocytes from patients with non-lethal JEB had a disorganized actin cytoskeleton, lacking focal contacts in the lamellipodia at the lateral edge of the substrate-associated cell surface [38]. BPA was confined to the central areas of the substrate-associated cell surface corresponding with immature hemidesmosome-like structures. Carter et al found that adherence of cultured keratinocytes to the substrate by focal contacts is mediated by the integrin α3β1 adhesion receptor, encircling α6β4 containing hemidesmosome-like structures [39]. It has recently been claimed that epiligrin is the putative ligand of α3β1 and it appears to locate underneath adhesion complexes composed of α3β1 focal contacts and α6β4 hemidesmosome-like structures [39]. In vivo, basal keratinocytes might, as in the in vitro situation, adhere to the basement membrane by focal contacts at the cell periphery. The multiple molecular targets re-

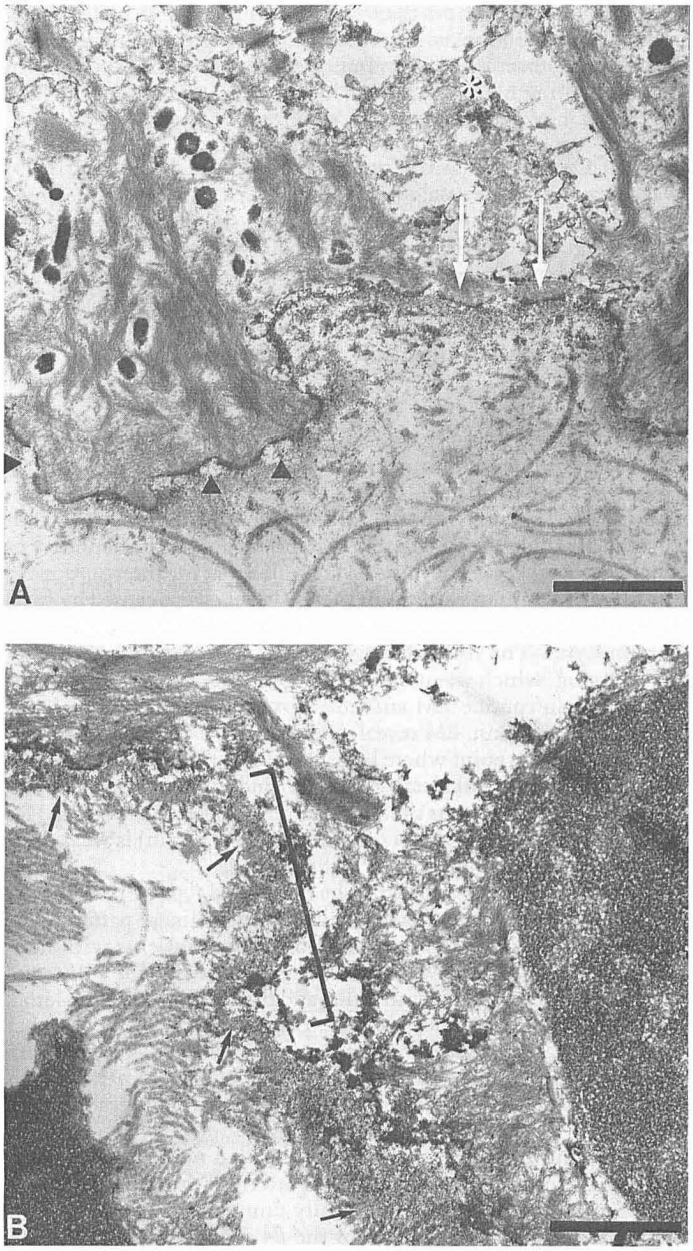

Figure 5. Immunoelectron microscopic detection of $\beta 4$ (450-10D) in the basement membrane zone of two adjoining basal keratinocytes in (*A*) a healthy human control and in (*B*) a patient with lethal JEB (patient 2). (*A*) The reaction product is present in a discontinuous hemidesmosome-associated linear distribution pattern along the basal surface of keratinocytes. Some tissue damage is present between the keratinocytes (*asterisks*) due to freezing, but the lamellipodium at the basal boundary remains attached to the lamina densa (*arrows*). The morphology of the areas between (*arrowheads*) hemidesmosomes has suffered from freezing and thawing. (*B*) The reaction product is interrupted along the basement membrane between keratinocytes. The adjoining basal lamellipodia have been disrupted (*bar*) from the lamina densa due to freezing and thawing. The lamina densa has remained intact (*arrows*). *Bar*, 1 μm.

sponsible for keratinocyte adhesion might explain the clinical and morphologic heterogeneity in the JEB subgroup [1].

The split found along the basement membrane zone of hair follicles in patients with lethal JEB separated the extracellular from the intracellular $\alpha 6\beta 4$ epitopes, apparently dividing the integrin. In contrast, the split in NaCl-incubated skin from healthy human controls almost completely occurred beneath all the $\alpha 6\beta 4$ epitopes, as also found by others [23,35]. It thus appears that the spontaneous split in JEB skin is localized more towards the cell membrane of the keratinocyte than the split induced by NaCl-incubation of healthy human control skin, suggesting a defect in the $\alpha 6\beta 4$ itself or perhaps of its ligand.

A possible molecular abnormality in JEB might be the absence of the ligand for the integrin $\alpha 6\beta 4$ or another important entity involved in the integrin-ligand interaction. The $\beta 4$ subunit is focally overexpressed in the cytoplasm of basal keratinocytes of patients with lethal JEB, which might be the result of retention or overproduction of the receptor because of a lack of the extracellular ligand. The extracellular ligand for the $\alpha 6\beta 4$ receptor has not been identified, but it probably is a protein of the basement membrane. In contrast to most integrins, $\alpha 6\beta 4$ appears to recognize a binding sequence in its extracellular ligand that is different from the classical Arg-Gly-Asp (RGD) [21]. One possible explanation for the coincidence of the absence of GB3 with an altered distribution of $\alpha 6\beta 4$ is that the antigen (nicein) recognized by GB3 is the ligand for $\alpha 6\beta 4$. Verrando et al hypothized that nicein might be an iso-laminin because of its discrete homologies with laminin [40]. In the same respect, $\alpha 6\beta 4$ might be the receptor for an iso-laminin, although $\alpha 6\beta 4$ does not bind to either mouse laminin fragment E8 or human laminin affinity columns, as the classical laminin receptor integrin $\alpha 6\beta 1$ does in many different cell types [41,42]. The tissue distribution of GB3 [33] (skin, larynx, esophagus, stomach, small intestine, colon, bladder, pancreas) is similar to that of the $\beta 4$ subunit [43], except that the latter is also found in bloodvessels ([44], this study]) and in peripheral nerves ([43], this study). However, multiple forms of $\beta 4$ may exist, possibly due to cell-type specific alternative mRNA splicing [45–47]. Further support for the possible relation between GB3 and $\beta 4$ is that neither occur in melanocytes [21,33,48]. However, evidence for a direct interaction between $\alpha 6\beta 4$ and nicein has to be determined. Other possible ligands for $\alpha 6\beta 4$ are 19-DEJ-1 [4], 125 kD mABHD [37], 1-2B7B [49], or the recently identified proteins kalinin [50] and epiligrin [39], which all preferentially localize below the sub-basal dense plate of hemidesmosomes.

The results of this preliminary study of two patients with lethal JEB need further confirmation in other patients. Immunohistochemical studies of cultured JEB keratinocytes may also shed more light on the relationship between abnormal adhesion, lack of ligands, and redistribution of hemidesmosomes in this hereditary bullous disorder.

We conclude that the expression of the integrin $\alpha 6\beta 4$ and of BPA is unreduced in junctional and dystrophic EB. However, in skin from patients with lethal JEB, who lack expression of GB3, immunohistochemical staining of integrin $\alpha 6\beta 4$ and BPA is interrupted at the peripheral border of basal keratinocytes due to freeze induced partial cell detachment from the basement membrane.

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